

COMPOSITION AND PROCESS FOR STABILIZING OF BIOMOLECULES

The present invention relates generally to the field of biotechnology. In particular, the invention relates to a composition and process for stabilizing, or, as the case may be, preserving biological molecules as well as devices that comprise the correspondingly stabilized biomolecules.

The use of proteins and polypeptides in industrial products and processes requires large quantities of these biomolecules, in particular for clinical/diagnostic and pharmaceutical purposes. Whereas the required basic techniques such as isolation and purification of proteins in industrial quantities are mostly established, the most difficult aspect of the use of such biomolecules resides in maintenance for the envisioned application, of the desired native, or, active molecular properties. Particularly, in the storage and transport of biomolecules, losses in activity often have to be taken into account, whereby the success of later applications is put in risk. Commercially obtainable protein preparations therefore for the most part comprise compounds whose presence can minimize the activity loss during storage or transport. Furthermore, storage and transport is mostly carried out under low temperatures, although a freezing of, for example, particular proteins may be undesirable because of molecular changes.

It is known from the literature that particular plants and animals have developed mechanisms to survive in the state of approaching complete dehydration. This state of stress is referred to anhydrobiosis and is observed in organisms that are exposed to dry conditions. During anhydrobiosis, the organism finds itself in a kind of resting state until a rehydration allows normal metabolism to continue. Common characteristic property of these organisms is the synthesis of high concentrations of non-reducing sugars that is induced during anhydrobiotic conditions. The observed accumulation of large quantities of trehalose as a response to dehydration in various organisms leads to a protection of membranes and proteins from damage to their molecular integrity and correlates to a certain tolerance with respect to water removal. One assumes that the sugar replaces, or, as the case may be, functionally substitutes the removed water molecules and is involved in the formation of an intracellular organic glass by which, one assumes, the cell contents are stabilized.

In the state of the art, the use of trehalose in the production of antibody-coated microtiter plates is described in order to stabilize this normally very quickly

denatureable protein species (V.K. Nguyen et al., Protection of immunoreactivity of dry immobilized proteins on microtitration plates in ELISA: application for detection of autoantibodies in *Myasthenia gravis*, *J. of Biotechnology*, 72, pp. 115 to 125 (1999)). In this case, microtiter plates having antibodies immobilized thereon are covered with a bovine serum albumin (BSA) and trehalose-containing film and are thereafter dried. The immobilized dried antibodies of the ELISA plates made in this manner showed a storage capability of up to thirty days even at increased temperatures (up to 50° C).

An increase of the storage capability, particularly from a commercial viewpoint, at room temperature or even tropical conditions can, however, not be achieved with this technology. Particularly in connection with the preparation of large quantities of immobilized proteins, it is desirable to have a storage capability for a time period of over a year with substantially constant biological activity, or functionality of the biomolecule.

A further disadvantage of the above-described technology lies in the use of bovine serum albumin (BSA), a protein mixture of which it is known that, in the framework of antibody-aided applications, unspecific binding with antibodies results and thereby creates undesired cross reactions through which the entire experimental result is negatively influenced.

It is known, particularly from plant seeds and pollen that, in reaction to water removal, proteins of the LEA class are formed in them. (LEA = 'late embryogenesis abundant') (J. Ingram and D. Bartels, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 47, pp. 377-403 (1996)). The LEA proteins also identified in nematodes, comprise a special, 11 amino acid motif, which presumably forms an amphipathic α -Helix through which the oligomerization of the protein is controlled. The LEA proteins are extremely hydrophilic and resistant to denaturation by heat. Initial experiments with a protein of this class purified from the pollen of *Typha latifolia* have shown that, in vitro, sucrose glasses can be stabilized by incubation with this protein. It is therefore suspected that non-reducing sugars and representatives of the protein class LEA work together in a synergistic manner in the formation of a stable bioglass in the cytoplasm of anhydrobiotic plants and in seeds and pollen being resistant to desiccation (J. Brown et al, Plant desiccation gene found in a nematode, *Nature*, 416, p. 38 (2002)).

An object of the present invention is therefore the provision of compositions and processes for stabilizing, or, as the case may be, preserving biomolecules, by which the disadvantages of the state of the art are overcome, and with which the desired biological activity of the molecules can be preserved, even without cooling, over a longer period of time.

The object is solved according to the present invention by the composition according to the main claim.

The composition according to the present invention comprises at least one non-reducing disaccharide, selected from the group consisting of trehalose (D-glucopyranosyl-D-glucopyranose), sucrose (β -D-fructofuranosyl- α -D-glucopyranoside), as well as derivatives thereof, and at least one protein or polypeptide of the LEA class.

According to a preferred embodiment, the composition according to the present invention comprises trehalose as well as at least one protein or polypeptide of the LEA class with an 11 amino acid motif, characterized by the following general formula (SEQ ID NO. 1):

(1)-(2)-(3)-(4)-(5)-(6)-(7)-(8)-(9)-(10)-E,

wherein

- (1) signifies K or T,
- (2) signifies A, G, K, M or T,
- (3) signifies R, D, A, E, Q or K,
- (4) signifies E, K or S,
- (5) signifies T, F, Y or A,
- (6) signifies K, R, T or A,
- (7) signifies D, E or Q,
- (8) signifies S, R, Y or K,
- (9) signifies A or T, and
- (10) signifies G, A or R.

In a particularly preferred embodiment, the composition comprises at least one protein or polypeptide of the LEA subclass 3 having an amino acid sequence that is coded by a nucleotide sequence as deposited in the GenBank under the accession number AF423069 or S39475.

The composition according to the present invention comprises, as particularly preferred, at least one protein or polypeptide of the LEA subclass 3 with an 11 amino acid motif, selected from the group consisting of:

- (a) K-T-A-E-F-R-D-S-A-G-E (SEQ ID NO. 2),
- (b) K-G-Q-E-F-K-E-R-A-G-E (SEQ ID NO. 3),
- (c) K-A-E-E-T-K-Q-R-A-G-E (SEQ ID NO. 4),
- (d) K-M-D-E-T-K-Q-R-A-G-E (SEQ ID NO.5),
- (e) K-A-R-K-T-K-D-S-A-A-E (SEQ ID NO. 6),
- (f) K-A-K-E-Y-K-D-Y-T-A-E (SEQ ID NO. 7),
- (g) K-A-R-E-T-T-E-K-A-R-E (SEQ ID NO. 8), and
- (h) T-K-D-S-A-A-E-K-A-R-E (SEQ ID NO. 9).

According to a preferred embodiment, the composition comprises the components of the non-reducing disaccharide and the protein or polypeptide of the LEA class in respective quantities of from 0.01 to 15, or, as the case may be, 0.00001 to 1 weight percent, in each case with reference to a ready-to-use solution.

For example, the composition is produced by adding 0.1 weight percent of a purified LEA protein and 5 weight percent trehalose to a solution of 50 mM phosphate and 100 mM NaCl with a pH value of 6.8. If desirable, further components such as, for example, sodium azide (0.02 weight percent) can also be added.

It is clear to one of skill in the art that by means of the sequence and motif information homologues representatives of the protein class LEA from different sources can be obtained and applied according to the present invention. As follows, all homologs are included, so far as they can stabilize a biological glass formed from non-reducing sugars, such as, in particular, trehalose and/or sucrose.

Exemplary representatives of the protein class LEA are presented in the following table, however these examples do not limit the present invention. The respective sequence information can easily be obtained by one of skill in the art by means of the presented data.

Data bank	Organism	Reference (Accession No.)
SWISS-PROT	Gossypium hirsutum	P09422
SWISS-PROT	Raphanus sativus	P21208
SWISS-PROT	Zea mays	P46517

SWISS-PROT	Hordeum vulgare	Q02400
SWISS-PROT	Hordeum vulgare	Q05191
SWISS-PROT	Hordeum vulgare	Q5190
SWISS-PROT	Hordeum vulgare	P46532
SWISS-PROT	Helianthus annuus	P46515
SWISS-PROT	Helianthus annuus	P46515
SWISS-PROT	Gossypium hirsutum	P09411
SWISS-PROT	Gossypium hirsutum	P46518
SWISS-PROT	Gossypium hirsutum	P09443
SWISS-PROT	Gossypium hirsutum	P13940
SWISS-PROT	Gossypium hirsutum	P09444
SWISS-PROT	Gossypium hirsutum	P46521
SWISS-PROT	Gossypium hirsutum	P4522
SWISS-PROT	Brassica napus	P13934
SWISS-PROT	Gossypium hirsutum	P13939
SWISS-PROT	Zea mays	Q42376
SWISS-PROT	Tricicum aestivum	Q03968
TrEMBL	Phaseolus vulgaris	O24442
TrEMBL	Arabidopsis thaliana	O64820
TrEMBL	Arabidopsis thaliana	O65148
TrEMBL	Arabidopsis thaliana	O80576
TrEMBL	Arabidopsis thaliana	O81483
TrEMBL	Oryza sativa	P83196
TrEMBL	Oryza sativa	P83197
TrEMBL	Glycine max	P93165
TrEMBL	Glycine max	Q01527
TrEMBL	Tricicum aestivum	Q03967
TrEMBL	Arabidopsis thaliana	Q39138
TrEMBL	Citrus sinensis	Q39466
TrEMBL	Chlorella vulgaris	Q39660
TrEMBL	Gossypium hirsutum	Q39793
TrEMBL	Gossypium hirsutum	Q39797

TrEMBL	Glycine soja	Q39919
TrEMBL	Onoclea sensibilis	Q40697
TrEMBL	Picea glauca	Q40842
TrEMBL	Picea glauca	Q40843
TrEMBL	Picea glauca	Q40848
TrEMBL	Picea glauca	Q40858
TrEMBL	Picea glauca	Q40869
TrEMBL	Zea mays	Q41804
TrEMBL	Oryza sativa	Q8S4X7
TrEMBL	Brassica campestris	Q8S8Z2
TrEMBL	Brassica napus	Q8S8Z2
TrEMBL	Arabidopsis thaliana	Q96273
TrEMBL	Oryza sativa	Q9AWZ5
TrEMBL	Arabidopsis thaliana	Q9FG31
TrEMBL	Arabidopsis thaliana	Q9FK14
TrEMBL	Arabidopsis thaliana	Q9FK15
TrEMBL	Arabidopsis thaliana	Q9FKV7
TrEMBL	Oryza sativa	Q9FPB2
TrEMBL	Arabidopsis thaliana	Q9LF88
TrEMBL	Oryza sativa	Q9LGL8
TrEMBL	Arabidopsis thaliana	Q9LT74
TrEMBL	Euphorbia esula	Q9M556
TrEMBL	Arabidopsis thaliana	Q9S7S3
TrEMBL	Onoclea sensibilis	Q9S2B2
TrEMBL	Arabidopsis thaliana	Q9SKPO
TrEMBL	Chlorella vulgaris	Q9SLP7
TrEMBL	Arabidopsis thaliana	Q9XID7
TrEMBL	Arabidopsis thaliana	Q9ZPW6
TrEMBL	Glycine max	Q9ZTZ2

The LEA proteins or polypeptides proposed according to the present invention can be isolated from natural sources, produced recombinantly or synthesized. The processes to be used are well known to one of ordinary skill in the art.

A further aspect of the present invention relates to the provision of a method for stabilizing or preserving biomolecules in which the molecules to be protected are incubated in the composition according to the present invention. After a sufficient period of incubation, the preparation can then, for example, be dried at room temperature and stored until use without cooling. Insofar as the process is used for stabilization of biomolecules immobilized on particular surfaces, these loaded surfaces are covered with the composition according to the present invention. This can, for example, take place by spraying the composition on the surface or by immersing the surface in the composition. In the case of an immersion method, the surface should, preferably, be withdrawn with a speed of about 2 mm per second so that a uniform wetting with the composition according to the present invention can take place.

A further aspect of the present invention relates to the provision of surfaces that have been covered with the composition of the present invention. Biomolecules are directly or indirectly immobilized on preferred surfaces and are stabilized or preserved with the composition of the present invention. Particularly preferred surfaces are components of analytical and/or diagnostic devices, such as, for example, biochips, sensor chips, microtiter plates, test tubes, and the like. The material of the surface is not limited and can, for example be selected from glass, quartz glass, quartz, silicon, polymers (PMMA, polystyrene, polyethylene, polypropylene, PVC, etc.), and membranes such as, for example, nitrocellulose, nylon and microfiber membranes, as well as paper.

The presently used term "biological molecule" and "biomolecule" encompass any substances and compounds substantially of biological origin that have properties that are relevant within the framework of scientific, diagnostic and/or pharmaceutical applications. Encompassed are not only native molecules, such as those that can be isolated from natural sources, but also forms, fragments and derivatives derived therefrom, as well as recombinant forms and artificial molecules, as long as at least one property of the native molecules is present. Preferred biomolecules are those that can be applied for analytical, diagnostic and/or pharmaceutical purposes, such as nucleic acids and their derivatives (DNA, RNA, PNA, LNA, ribozymes, oligonucleotides, plasmids, chromosomes), peptides and proteins (enzymes, receptor proteins, protein complexes, peptide hormones, antibodies), as well as biologically

active fragments thereof, carbohydrates and their derivatives such as, in particular, glycosylated proteins and glycosides, and fats, fatty acids and lipids.

It is clear to one of skill in the art that the composition and processes according to the present invention can also be applied to cellular tissues and complete cells as well as portions thereof (organelles, membranes and membrane fragments, etc.) as long as they are carriers of the above biomolecules. For this reason, tissues, cells and portions thereof are basically encompassed by the term "biomolecules".

The used terms "stabilization" and "preservation" relate to the structural or functional integrity of biomolecules and the biological properties based thereon. The required activity of a biomolecule for a particular application requires, for example, the extensive maintenance of its primary, secondary and/or tertiary structure. The biological activity of a nucleic acid probe comprises, for example, its property for forming a hybridization complex with a nucleic acid target which is complementary to the probe. The biological activity of an antibody comprises, for example, a specific binding of its antigen.

The invention is more closely elucidated by means of the following example.

Coating of Surfaces

The formation of a composition according to the present invention results by dissolving 0.1% (weight/volume) recombinant LEA-protein and 5% (weight/volume) trehalose and 100 ml phosphate buffer (50 mM phosphate, 100 mM NaCl, pH 6.8). Subsequently, 0.02% (weight/volume) sodium azide is added as a cytotoxic agent against microbial impurities. After sterile filtration of the solution obtained using a 0.2 micrometer filter, the result is stored in an autoclaved sterile bottle.

In order to layer suitable surfaces according to the present invention, biochips (for example microscope slides) with immobilized biomolecules are immersed in the solution in a clean room (or sterile work bench) and are drawn out of the solution with a speed of 2 mm per second. In this manner a thin layer of the trehalose/LEA solution is dried on the chip and leads to the stabilization of the biomolecules.

For storage, the chips with stabilized biomolecules treated in this manner can be packaged in plastic bags and stored at room temperature under an atmosphere of nitrogen. The bags can preferably be packed in a carton sealed from light in order to prevent possible photo degradation of the proteins.

For use, the chips are taken from their packaging and rehydrated (5 minutes, RT) with assay buffer (for example PBS buffer). Next, the sample liquid is incubated directly on the chip and the assay is carried out. All kinds of receptor ligand interactions known in the art can be carried out. If desired the removal of the stabilizer can also be left out. In this case the sample is directly put onto the trehalose layered surface. This is above all practical if it has been previously shown that neither trehalose nor LEA protein interfere with the detection.